PRELIMINARY AMENDMENT
NS Entry of PCT/EP2003/012839

AMENDMENTS TO THE SPECIFICATION

Please amend the specification at page 1, after the title and before line 4, by inserting:

This Application is a 371 of PCT/EP2003/012839, filed November 17, 2003; the disclosure of which is incorporated herein by reference.

Page 3, lines 4-18, are amend as follows:

Prideaux ~~(~~(The 16th International Pig Veterinary Society Congress, Melbourne (Australia) 17-20th September 2000, pag.439-442)) describes a vaccine prepared from a strain with an inactivated *apx*IIC gene that secretes and express a non-activated *Apx*II toxin unable therefore to attach to the target cells.

So, the live attenuated vaccines described in the previous background of the invention, based on App strains without haemolytic capability, are less immunoprotective because they have suffered modifications in their structure that do not allow them to attach to the membrane receptor of the target cells. Furthermore these can not generate antibodies against *Apx*I and/or *Apx*II toxins, since these are not secreted by the cell. Frey et al. (Gene ~~(~~142:97-102 (1994)) describe the amino-acid sequence of the ApxI exotoxine from a serotype I strain and Smiths et al.;~~((~~Infection and Immunity 59:4497-4504 (1991)) describe the amino-acid sequence of the ApxII exotoxin of a serotype 9 strain.

Page 3, lines 22-27, are amended as follows:

The authors of the present invention have discovered a method to obtain an immunogenic and non-haemolytic App strain from an App virulent strain which has been modified in at least one segment of *apx*IA gene (SEQ ID NO 1) and optionally in a segment of the *apx*IIA gene (SEQ ID NO 2) which code a

transmembrane domain of the Apx cytolytic and haemolytic exotoxins.

Page 4, lines 24-33, are amended as follows:

Fig. 1 shows an alignment performed with the ClustalX program (Thompson et al; Nucleic acid Research **24**:4876-4882 (1997)) between the amino-acid sequences of ApxI coming from a serotype 1 strain ┼(Frey et al; Gene **142**: 97-102 (1994)) and ApxII from serotype 9 ┼(Smits et al.; Infection & Immun. **59**:4497-4504 (1991)). In this figure only the sequence contained between amino-acids 1 to 594 of ApxI and 1 to 590 of ApxII have been enclosed. On the alignment the following regions are framed: H1 (amino-acids 233 to 253), H2 (amino-acids 296 to 315) and H3 (amino-acids 369 to 405). These regions correspond respectively to the three transmembrane domains present in both Apx.

Page 5, line 26 to Page 6, line 8, are amended as follows:

Figure 3 is divided in three panels: Panel A shows the restriction maps in kilobases (kb) and the distribution of the genes in the operon *apx*I from the genome of App. In light gray, the *apx*IA gene is depicted, being the target of the different recombination events, and in dark gray the adjacent genes *apx*IC, *apx*IB and *apx*ID. The different genes or regions of plasmid pApxIΔH2 are drawn using skewed bars. The coding fragments of the transmembrane helices (H1, H2 and H3) of apxIA are highlighted in Black. The names and some detailed structures in figure 2 plasmids have been simplified. Thus gfpUV comprises the ptac promoter and the atpE/GFPUV fusion; OriV indicates the vegetative origin of replication of R6K and OriT the origin of transference by conjugation of RP4. In (1) and (3) both are shown the restriction map obtained with

enzyme *Xho*I and the distribution of the genes of operon *Apx*I of the App genome are shown. In (2) the restriction map of the same operon after the insertion of plasmid pApxIΔH2 in the App genome is shown. This insertion occurs by a unique homologous recombination event between flanking regions 5´ of H2 placed in plasmid pApxIΔH2 and the App genome respectively.

Page 10, lines 5-17, are amended as follows:

The transmembrane domains, present in the *apx*IA and in *apx*IIA genes of the haemolytic and cytolytic exotoxins, were detected using the Transmem and Helixmem programmes above mentioned. The prediction performed on the amino-acid sequences of the haemolytic and cytolytic exotoxins ApxI and ApxII indicate that the transmembrane domains, also named transmembranes, are found located in the following zones of the sequence of the exotoxins:

- First transmembrane domain H1: between amino-acids 233 and 253 corresponding to the nucleotides 697~~699~~ to 759 from *apx*I.
- Second transmembrane domain H2: between amino-acids 296 and 315, corresponding to nucleotides 886~~888~~ to 945 from a*px*I
- Third transmembrane domain H3: between amino-acids 369 to 405, corresponding to nucleotides 1105~~1107~~ to 1215 from *apx*I

Page 10, lines 21-29, are amended as follows:

The modification is carried out, preferably, by deletion of the nucleotides 886~~885~~ to 945~~944~~ of the *apx*IA gene which code the second transmembrane domain of the App *Apx*I exotoxin.

Another preferred realization, of the method object of the present invention, furthermore introduces an additional deletion in the segment of *apx*IIA gene which codes the second

transmembrane domain of the ApxII exotoxin of App. Preferably a deletion of nucleotides 886~~885~~ to 945~~944~~ of gene *apx*IIA, which code the second transmembrane domain of the App *Apx*II exotoxin.

Page 12, lines 18-27, are amended as follows:

In the present invention the obtained mutant AppApxIH2$^-$ is identical to the original wild type App strain, except for the deletion of nucleotides 886~~885~~ to 945~~944~~ (both inclusive) of the coding sequence of gene *apx*IA which corresponds with that absence of the amino-acids 296 to 315 (both inclusive) in the produced ApxI.

In the present invention the obtained mutant AppApxI/IIH2$^-$ is identical to strain AppApxIH2$^-$ except by the deletion of nucleotides 886~~885~~ to 945~~944~~ (both inclusive) of the coding sequence of gene *apx*IIA which corresponds with the absence of amino-acids 296 to 315 (both inclusive) in the ApxII produced.

Page 13, lines 18-30, are amended as follows:

Another object of the invention is an App strain characterized because it has a deletion in nucleotides 886~~885~~ to 945~~944~~ of the *apx*IA gene, that code the second transmembrane of the ApxI exotoxin, deposited in the Colección Española de Cultivos Tipo (Spanish Collection of Type Cultures) with the registration number CECT 5985, according to the Treaty of Budapest of 28th April 1977, or a mutant thereof.

Another object of the invention is an App strain characterized by having a deletion of nucleotides 886~~885~~ to 945~~944~~ of the *apx*IA gene that code the second transmembrane of the ApxI exotoxin and besides a deletion of nucleotides 886~~885~~ to 945~~944~~ of *apx*IIA gene that code the second transmembrane of the ApxII exotoxin deposited in the Spanish Collection of Type

Cultures with the registration number CECT 5994, according to the Budapest Treaty, or a mutant thereof.

Page 15, lines 4-16, are amended as follows:

The techniques and DNA recombinant methods applied as follows, are described in detail in Sambrook and Russell (In Molecular cloning 3rd Ed. Cold Spring Harbor Laboratory Press, Cold spring Harbor New York (2001) and Ausubel et al; *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. (1998)). All PCR products were previously cloned in a pBE plasmid before being digested with restriction enzymes. This plasmid is a derivative of pBluescript SK2 (Stratagene) vector and presents the multiple cloning site substituted by a small nucleotidic sequence which specifies only the target of the restriction enzyme *Eco*RV.

The *E. coli* XL1-blue strain (Stratagene) has been used as a host for hybrid vectors based on plasmids pUC118 or pBluescript SK. The *E. coli* S17-1 λ pir strain (†Simon et al; Biotechnology **1**:784-791 (1983)) has been used as a host of the hybrid vectors based in plasmid pGP704.

Page 15, line 32 to Page 16, line 10, are amended as follows:

The three transmembrane domain which adopt an α- helix structure, were determined by means of the use of programmes TransMem †(Aloy *et al*; Comp. Appl. Biosc. **13**:213-234 (1997)) and Helixmem †(Eisenbeg *et al*; J. Mol. Biol. 179: 125-142 (1984)) as described for *E. coli* †(Ludwig *et al*; Mol. Gene. Genet. **226**:198-208 (1991)) applied to the amino-acid sequence of the ApxI coming from a serotype 1 strain †(Frey *et al*; Gene **142**: 97-102 (1994)) and the ApxII of a type 9 serotype †(Smits *et al*; Infection and Immunity **59**:4497-4504 (1991)). These programmes detected three regions which could act as

transmembrane helices in both proteins (Fig 1): the first transmembrane is located between amino-acids 233 and 253 (H1); The second transmembrane is located between amino-acids 296 and 315 (H2) and the third transmembrane between amino-acids 369 and 405 (H3) all of them from ApxI.

Page 16, lines 20-26, are amended as follows:

Plasmid pGP704 ~~(~~Miller and Mekalanos; J. Bact. **170**:2575-2583 (1988)) was cut simultaneously with restriction enzymes *Bgl*II and *Eco*RI. Using electrophoresis in agarose gel a 3.7 kb DNA fragment was isolated. This fragment incubated in a ligation reaction together with oligonucleotides pGP5´ (GAT CGA ATT CAG GAT ATC ACA GAT CT) (SEQ ID NO ~~1~~3) and pGP3´ (ATT TAG ATC TGT GAT ATC GTG AAT TC) (SEQ ID NO ~~2~~4). The obtained recombinant plasmid was named pGP1.

Page 16, line 32 to Page 17, line 17, are amended as follows:

Using plasmid pMAL-p2 (New England Biolabs) the sequences corresponding to promoter ptac were amplified by PCR using the ptac5´oligonucleotide primers (GAA TTC AAT GCT TCT GGC GTC AG) (SEQ ID NO ~~5~~3) and ptac3´ (GGT ACC GGA TGA GAT AAG ATT TTC) (SEQ ID NO ~~6~~4) which enclose respectively the restriction targets *Eco*RI and *Kpn*I in its 5´ends. Also from pMAL-p2 plasmid, by PCR the sequences corresponding to the rho-independent terminator of operon *rrn*B were amplified using the primer oligonucleotides *rrn*B5´ (GGT ACC GGA TGA GAT AAG ATT TTC) (SEQ ID NO ~~7~~5) and *rrn*B3´ (GAA TTC AAG AGT TTG TAG AAA CGC) (SEQ ID NO ~~8~~6) which enclose respectively the restriction targets *Kpn*I and *Eco*RI in their 5´ ends. The size of the DNA amplified fragment comprises 278 base pairs (bp).

With the plasmid pAG408 ~~(~~Suarez *et al*; Gene **196:** 69-74 (1997)) a fusion of the gene of the GFPUV protein with the

region that links to the ribosome of the *atp*E gene was amplified using the primer oligonucleotides GFP5´ (GGT ACC TAA TTT ACC AAC ACT AC) (SEQ ID NO 9~~7~~) and GFP3´ (GGT ACC TTA TTT GTA GAG CTC ATC) (SEQ ID NO 10~~8~~) which encloses the restriction target *Kpn*I in its 5´ends. The amplified fragment has a size of 830 bp.

Page 17, line 30 to Page 18, line 22, is amended as follows:

At this stage, the first objective was to obtain a DNA fragment contiguous to the 5´ end of the coding fragment of the second transmembrane helix of *apx*IA gene. Therefore a fragment of 897 bp was amplified by PCR from the purified geneomic DNA of the App strain HP816 using as primers the ApxIa5´oligonucleotides (GAT ATC ATG GCT AAC TCT CTC AGC TCG ATA G) (SEQ ID NO 11~~9~~) and ApxIa3´ (CTC GAG GCC TGC CGC CAC ACG TTG) (SEQ ID NO 12~~10~~), which enclose the restriction targets *Eco*RV and *Xho*I in its respective 5´ends. The 7th base of the oligonucleotide ApxIa5´ (SEQ ID NO 9) corresponds with the first base of the start codon of the translation of *Apx*Ia gene. The seventh base of oligonucleotide ApxIa3´ (SEQ ID NO 10) is complementary to the 885 base of the coding sequence of gene *apx*IA, being the latter the last base before the initiation of the sequence for the second transmembrane helix.

The second objective of this phase was to obtain a DNA fragment contiguous to the 3´end of the coding segment of the second transmembrane helix of *apx*IA gene. Therefore, using PCR, a fragment of 1042 bp was amplified from purified genomic DNA of the strain App HP816 using as primers the oligonucleotides ApxIb5´ (CTC GAG CCG CTT TCG TTC TTA AAT GTT GCG) (SEQ ID NO 13~~11~~) and ApxIb3´ (AGA TCT TCA CCG GCT TTC TGT GCA CTT TG) (SEQ ID NO 14~~12~~) which include the restriction

targets *Xho*I and *Bgl*II in its respective 5´ ends. The 7th base of oligonucleotide ApxIb5´ (SEQ ID NO 13~~11~~) corresponds with the base 946~~944~~ of the coding sequence of the *apx*IA gene, being this one the first base after the end of the sequence for the second transmembrane helix. The seventh base of oligonucleotide ApxIb3´ (SEQ ID NO 14~~12~~) is complementary to base 1975 of the coding sequence of the gene *apx*IA.

Page 18, line 32 to Page 19, line 24, are amended as follows:

The first objective, at this stage, was to obtain a DNA fragment contiguous to the 5´ end of the coding segment of the second transmembrane helix of gene *apx*IIA. Therefore, by PCR, a 871 bp fragment was amplified from the purified genomic DNA of the App strain HP816 using as primers the oligonucleotides ApxIIa5´ (GAT ATC AAA TCG TCC TTA CAA CAA GGA TTG) (SEQ ID NO 15~~13~~) and ApxIIa3´ (GAA TTC ACC TGA AGC GAC TCG TTG GGC) (SEQ ID NO 16~~14~~) which enclose the restriction targets *Eco*RV and *Eco*RI in its 5´ respective ends. The number 7 base of oligonucleotide ApxIIa5´ (SEQ ID NO 15~~13~~) corresponds to base 27 of the coding sequence of the gene *apx*IIA. The seventh base of oligonucleotide ApxIIa3´ (SEQ ID NO 16~~14~~) is complementary to the base 885 of the coding sequence of gene *apx*IIA, being this one the last base before the start of the sequence for the second transmembrane helix.

The second objective of this step, was to obtain a DNA fragment contiguous to the 3´end of the coding segment of the second transmembrane helix of *apx*IIA gene. Therefore a 952 bp fragment was amplified by PCR from the purified genomic DNA from the App strain HP816 using as primers the oligonucleotides ApxIIb5´ (GAA TTC CCT CTT TCA TTC TTA AAT GTA GC) (SEQ ID NO 17~~15~~) and ApxIIb3´ (AGA TCT GCC ATC AAT AAC GGT

AGT ACT TG) (SEQ ID NO 1816), which enclose the restriction targets *Eco*I and *Bgl*II at its 5´ respective ends. The 7th base of oligonucleotide ApxIIb5´ (SEQ ID NO 1715) matches up with the base 946944 of the coding sequence of gene *apx*IIA, being the latter the first base after the end of the sequence for the second transmembrane helix. The seventh base of the oligonucleotide ApxIIb3´ (SEQ ID NO 1816) is complementary to the base 1845 of the coding sequence of the *apx*IIA gene.

Page 26, lines 20-25, are amended as follows:

The recombinant so obtained, characterized by having a deletion in nucleotides 886885 to 945944 in the *apx*IA gene which code the second transmembrane domain of the *Apx*I exotoxin, has been named AppApxIH2$^-$. On the 10th January 2002 this has been deposited in the Colección Española de Cultivos Tipo with the registration number CECT 5985, according to the conditions established in the Budapest treaty.

Page 28, lines 2-9, are amended as follows:

The recombinant strain so obtained has been renamed AppApxI/IIH2$^-$. This is characterized by having a deletion in nucleotides 886885 to 945944 in the *Apx*IA gene which code a second transmembrane domain of the ApxI exotoxin and furthermore a deletion of nucleotides 886885 to 945944 of the *apx*IIA gene which codes the second transmembrane domain of the *Apx*II exotoxin. This strain has been deposited in the Coleccion Española de Cultivos Tipo, on the 12th June 2002 with the registration number CECT 5994 as specified in the conditions of the Budapest Treaty on patents.

Page 30, line 13 to Page 31, line 17, are amended as follows:

Two out of 5 animals of this group died during this period of time. At the necropsy, four out of five animals, showed severe lung lesions. The animals which had been inoculated with the strain AppApxIH2$^-$ showed also a modification of their behaviour, although these signs slowed down from the forth inoculation day. The clinical signs were milder and were only observed in 50% of the pigs. Although none of the animals of this group died during the trial, 70% of them showed lesions at the necropsy although all of them were found to be milder than the previous group. The third group was inoculated with the strain AppApx/IIH2$^-$. Although four of the animals showed mild modified behaviour, these slowed down from the 48th hour post-inoculation. The two animals that showed limited clinical signs also recovered within 48 hours after the inoculation. No lung lesions were observed in none of the animals at the necropsy. The assessment of the lung lesions was done according to Hannan et al; ~~+~~(Research in Veterinary Science **33**:76-88 (1982)). The values shown are the arithmetical means of each group together with the standard deviation. According to these results, the AppApxI/IIH2$^-$ strain is non-virulent and can be used safely as a live vaccine. It is important to highlight that the App strain inoculated was recovered in 80% of the pigs of this group, seven days after its administration. This result indicates that the viability of the AppApxI/IIH2$^-$ strain in an experimental infection is not modified in spite of the fact that it is devoid of haemolytic activity. This fact is important if we bear in mind that it is essential that the microorganism remains viable so that the

Apx exotoxins can be generated and freed. Without the production of the Apx exotoxins, the attenuated strain could not be used as live vaccine since this would be unable to induce an immune response which would protect the animal against future infections (Reimer et al; Microbial Pathogenesis **18**:197-209 (1995)). In all trials a strong immunogenic response has been achieved.